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Review Article

Endogenous Damage-Associated Molecular Pattern Molecules at the Crossroads of Inflammation and Cancer[1]

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Abstract

Inflammatory mediators play important roles in the development and progression of cancer. Cellular stress, damage, inflammation, and necrotic cell death cause release of endogenous damage-associated molecular pattern (DAMP) molecules or alarmins, which alert the host of danger by triggering immune responses and activating repair mechanisms through their interaction with pattern recognition receptors. Recent studies show that abnormal persistence of these molecules in chronic inflammation and in tumor microenvironments underlies carcinogenesis and tumor progression, indicating that DAMP molecules and their receptors could provide novel targets for therapy. This review focuses on the role of DAMP molecules high-mobility group box 1 and S100 proteins in inflammation, tumor growth, and early metastatic events.

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Introduction

A century and a half ago, German physician and pathologist Rudolph Virchow first reported that infectious diseases showed signs of a "tumor process" and that inflammatory cells were frequently present in tumor biopsies [1]. Noting similarities between wound healing and tumor stromal generation, Harold Dvorak referred to tumors as "wounds that do not heal" [2]. Although the association was largely ignored for many years, increasing evidence linking inflammation and tumorigenesis has triggered renewed interest in understanding the molecular and cellular mechanisms involved in cancer-related inflammation. Emerging information points to two different pathways linking the pathologies [3]: an extrinsic pathway mediated by chronic inflammation that increases the risk of tumorigenesis (inflammation-induced cancer) and an intrinsic pathway in which genetic alterations, in the absence of an underlying inflammation, initiate a tumor-driven host immune response leading to a tumor microenvironment composed of inflammatory cells (cancer-induced inflammation).

Prolonged subclinical inflammation and associated necrotic cell death also cause release of intracellular molecules that alert the immune system to danger, evoking responses leading to epithelial regeneration, angiogenesis, proliferation, and ultimately tumorigenesis. These events resemble normal injury-related stromal reactions and wound healing processes, which the tumor cells co-opt for growth, and further subvert to counteract normal regulatory immune responses. Recent studies show that these endogenous damage-associated molecular pattern (DAMP) molecules or alarmins, released from necrotic cells and activated leukocytes, play critical roles in both extrinsic and intrinsic pathways of cancer-related inflammation. These studies prompt a paradigm shift in understanding tumor development in adults as a pathological sequence initiated by cycles of inflammation and necrotic cell death [4,5]. In this review, we focus on the contribution of DAMP molecules high-mobility group box 1 (HMGB1) and proinflammatory S100 proteins to inflammation and cancer.

Inflammation and Cancer

Inflammation-Induced Cancer

It is estimated that infections and chronic inflammatory responses are involved in the pathogenesis of approximately 15% to 20% of

Abbreviations: DAMP, damage-associated molecular pattern; DMBA/TPA, 7,12-dimethyl benz[*a*]anthracene/12-*O*-tetradecanoylphorbol-13-acetate; HMGB1, high-mobility group box 1; IL, interleukin; MDSC, myeloid-derived suppressor cells; NF-κB, nuclear factor-κB; PAMP, pathogen-associated molecular pattern; PRR, pattern recognition receptor; RAGE, receptor for advanced glycation end products; STAT3, signal transducer and activator of transcription 3; TAM, tumor-associated macrophage; TLR, Toll-like receptor; TNFα, tumor necrosis factor α

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human tumors, including gastric, colorectal, bladder, and liver cancers [6–10]. Other causes of chronic inflammation, including mechanical, physical, and chemical injury as well as dysregulated immune responses to injury, also predispose to cancers [10,11]. Agents modulating inflammation, including aspirin and nonsteroidal anti-inflammatory drugs, decrease the incidence of cancers [12–15], providing strong evidence for the extrinsic pathway of inflammation-based cancers. In addition, experimental animal models such as the 7,12-dimethyl benz[*a*]anthracene/12-*O*-tetradecanoylphorbol-13-acetate (DMBA/TPA)–induced papillomas and azoxymethane/dextran sulfate sodium (DSS)–induced colon cancers offer valuable insights into the initiation and progression of inflammation-based cancers [16–19].

Inflammation is an immune response to infection and tissue injury, characterized by the release of a complex regulatory network of mediators all aimed at combating the infectious or noxious agent, repairing damaged tissue, and restoring homeostasis [20]. In chronic inflammation, this response is exaggerated or sustained. Long-term inflammation is thought to lead to cancer because of the dysplastic degeneration of repaired epithelium by the continuous release of reactive oxygen and nitrogen species, which cause DNA damage resulting in genomic instability and providing a proliferative advantage for cells carrying mutations [21,22]. Recent evidence also indicate that chemokines and proinflammatory cytokines such as tumor necrosis factor α (TNFα), interleukin 6 (IL-6), and IL-23 play pleiotropic roles in tumor progression [23–28]. Transcription factors such as nuclear factor-κB (NF-κB) and signal transducer and activator of transcription 3 (STAT3) are activated in many tumors and serve as important molecular links between inflammation and cancer [29–31]. Nuclear factor-κB activation seems to play a complex role because NF-κB inhibition also induces certain tumors [32–34], and these contradictions have been incompletely understood [35,36].

Cancer-Induced Inflammation

The intrinsic pathway of cancer-related inflammation is orchestrated by the tumor itself without initiation by prior inflammation. Epithelial cells transformed by activation of oncogenes, by inactivation of tumor-suppressor genes, or by chromosomal rearrangement [37] secrete factors that recruit inflammatory cells to the tumor enabling the buildup of a microenvironment. The cancer cell coevolves with its associated stroma, and the cellular composition, size of the stromal component, and amplitude of response vary according to the tumor type and other factors. The stromal cells include macrophages and other myeloid cells, mast cells, endothelial cells, fibroblasts, dendritic cells, natural killer cells, and T and B cells [38]. Depending on the composition of the cells in the microenvironment and stage of disease, tumor stromal cells can stimulate or inhibit tumor growth. Tumor-associated macrophages (TAMs) secrete cytokines, chemokines, lipid mediators, growth and angiogenic factors, and matrix metalloproteinases that contribute to tissue remodeling and angiogenesis and regulate the adhesion, invasion, and motility of the tumor cells [6,39–43]. Lymphocytes may play a dual role in tumor progression [42]. Cytotoxic T cells could destroy tumor cells directly or through antibody-dependent killing. Conversely, although $CD4^+$ and $CD8^+$ T cells are major components of tumor microenvironments, they are largely ineffective in controlling tumor growth owing to the presence of Tregs and myeloid-derived suppressor cells (MDSCs) that are capable of suppressing T-cell proliferation. Myeloid-derived suppressor cells are a heterogeneous population of immature myeloid cells, characterized by the expression of Gr-1, CD11b, and IL4Rα [44–47]. They are normally present in low numbers in blood and lymphoid organs, but accumulate excessively in tumors, blood, and lymphoid organs of tumor-bearing mice, and are also found in various human cancers. Myeloid-derived suppressor cells suppress activation of $CD4^+$ and $CD8^+$ T cells, inducing their anergy or deletion, and promote the induction of Tregs, thus serving to blunt the body's immune responses to tumor antigens [48–50]. Immature dendritic cells (DCs) expressing HLA-DR accumulate in human tumors and provide a mechanism for immune evasion through inefficient antigen presentation [51,52]. Tumors also orchestrate other mechanisms to escape immune surveillance of the host [38].

As discussed below, DAMP molecules seem to play important roles in both inflammation-induced cancer and cancer-induced inflammation.

Damage-Associated Molecular Pattern Molecules

About two decades ago, the late Charles Janeway proposed that the immune system has evolved to protect the host, not against any innocuous foreign antigen but against infectious pathogens, and postulated that receptors on antigen-presenting cells of the innate immune system recognize pathogen-associated molecular pattern (PAMPs). It is now well established that cells of the innate immune system sense PAMPs through pattern recognition receptors (PRRs) such as Toll and Toll-like receptors (TLRs), the NOD1-like receptors, mannose receptor, and other scavenger receptors, and retinoic acid–inducible gene-1–like receptors, stimulation of which initiates a range of host defense mechanisms [53–59]. However, Janeway's model did not explain why strong immune responses are elicited against tissue transplants, ischemia-related injuries, tumors, and autoimmune diseases, none of which involve microbial components. In 1994, Matzinger [60] postulated that the immune system not only responds to pathogens but also senses and responds to intracellular alarm signals arising from nonphysiological cell death, damage, or stress. It is now known that necrosis of healthy cells in response to inflammation, ischemia, or hypoxia within tumors in fact releases endogenous molecules that alert the immune system of danger [61,62]. In live cells, the preexisting danger signals are hidden; apoptotic cells that are sequestered and cleared by phagocytes do not release their intracellular contents unless there is secondary necrosis while necrotic cells lose membrane integrity causing release of intracellular contents. These endogenous danger signals are called alarmins, and together with PAMPs that are microbial in origin, they are referred to as DAMPs [63,64]. The so-called signal 0 events initiated by DAMPs promote early innate and adaptive immune responses mediated through distinct receptors but interlinked to pathways orchestrated by cytokine, chemokine, and other inflammatory mediators. This early response mediated by DAMPs is also called "sterile inflammation" because it is initiated in response to trauma, ischemia, and other tissue damage in the absence of pathogenic infection. The sequence of immune responses to injury is so robust and stereotypical that it is used by pathologists to date the time of tissue injury in autopsies. Recent studies suggest that radiotherapy and some chemotherapeutic agents may cause preapoptotic and apoptotic changes on cell surface of cancer cells with concomitant release of soluble mediators that trigger DC activation and antitumor immune responses [65,66]. These findings suggest that DAMP molecules could have both protumor and antitumor effects [67].

Apart from their release from necrotic cells, several DAMP molecules are also secreted from activated leukocytes in response to microbial components or cytokines [63]. The molecules lack secretion signals but they are actively secreted through by a nonclassical pathway. A recent study shows that this noncanonical secretion is mediated by

activated caspase-1, suggesting regulation by inflammasomes [68]. Hence, there are different mechanisms by which DAMP molecules are released: 1) passive release from necrotic cells, 2) pulsatile release from apoptotic cells in response to radio or chemotherapy, and 3) induced release from activated immune cells by a noncanonical pathway. Because DAMP molecules promote the expression of cytokines, which in turn induce expression of DAMPs, signaling events mediated by DAMPs provide for a feed-forward cycle of inflammatory, tissue repair, and regeneration responses, which, when uncontrolled, may lead to carcinogenesis.

Endogenous DAMP molecules or alarmins include several intracellular proteins, DNA, RNA, and nucleotides (reviewed in Rock and Kono [61]). They are expressed in different cell types and function in normal cellular homeostasis. They are localized in the nucleus and cytoplasm (HMGB1), cytoplasm (S100 proteins), exosomes (heat shock proteins), and extracellular matrix (hyaluronic acid). On the basis of their origin and mechanism of action, the proinflammatory DAMP molecules can be classified as those that directly stimulate cells of the innate immune system and those that generate DAMPs from other extracellular molecules [61]. Here, we focus on two DAMP molecules HMGB1 and proinflammatory S100 proteins. They are released extracellularly by aforementioned processes, bind to PRRs, proteoglycans, and carboxylated glycans, trigger immune responses, promote tissue regeneration, and are implicated in inflammation and cancer.

High-Mobility Group Box 1

HMGB1 is a member of the nonhistone, chromatin-associated high-mobility group family of proteins [69]. It is a highly conserved gene expressed by all eukaryotic cells. During normal cellular homeostasis, HMGB1 is localized predominantly to the nucleus. It binds to the minor groove of DNA and facilitates the assembly of site-specific DNA-binding transcriptional complexes [69]. Nuclear functions of HMGB1 are essential to life because HMGB1 knockout mice die within 24 hours of birth from hypoglycemia [70].

Extracellular Release of HMGB1. HMGB1 is passively released by all cells upon necrotic cell death [71,72]. However, it can also be secreted by macrophages and DCs by a nonclassic pathway in response to lipopolysaccharide, interferon-γ, and TNFα [73,74]. Cytokine-mediated releases of HMGB1 from pituicytes and enterocytes have also been reported [75]. Lipopolysaccharide–mediated HMGB1 release is regulated by its hyperacetylation [76], whereas TNFα-induced secretion seems to be mediated through phosphorylation [77]. As mentioned earlier, this active secretion is through leaderless, non–Golgi-dependent pathways. Outside the cell, HMGB1 behaves as a cytokine, promoting inflammatory responses [74]. Exciting new reports show that HMGB1 secreted by apoptotic tumor cells after chemotherapy or radiation therapy promotes antitumor responses [65]. In addition, apoptotic cells activate macrophages that engulf them to secrete HMGB1 [78].

HMGB1 and Inflammation. HMGB1 released from necrotic cells induces inflammation [71]. It induces DC maturation, migration, and T-cell activation [79–81]. Monocytes, T cells, and endothelial cells release cytokines and inflammatory mediators in response to HMGB1, all of which augment the local inflammatory environment [79,80,82–86]. Administration of HMGB1 to mice significantly increases serum TNFα levels [83]. Purified recombinant HMGB1 also induces a delayed and biphasic release (3 and 8-10 hours after stimulation) of TNFα, IL-1α, IL-1β, IL-6, IL-8, and macrophage inflammatory protein 1α and β from human monocytes at concentrations within the pathological range observed in sepsis [83]. This effect is restricted to monocytes because it does not release cytokines from lymphocytes. HMGB1 also induces TNFα, IL-1β, IL-6, and nitric oxide production by murine macrophages through receptor for advanced glycation end products (RAGE)–dependent signaling pathways [82] and IL-6, monocyte chemoattractant protein 1, and thrombin-antithrombin complex levels in peritoneal lavage fluid and plasma of mice through TLR4 and RAGE-dependent mechanisms [87]. It has been suggested that the HMGB1 polypeptide itself has a weak proinflammatory activity and that binding to bacterial components including lipids may strengthen its effects [84]. HMGB1 also acquires enhanced proinflammatory activity through binding to cytokines such as IL-1β [88]. Furthermore, HMGB1 promotes the expression of intercellular adhesion molecule 1 and vascular cell adhesion molecule 1 on the surface of endothelial cells [85,86]. It mediates hemorrhagic shock–induced NAD(P)H oxidase activation and NF-κB–dependent gene expression in neutrophils [89,90]. HMGB1-DNA complexes promote maturation of immune cells and production of cytokines [91,92] while suppressing immune response in a few cell types [93].

Wang et al. [94] first described proinflammatory cytokine activity of HMGB1, when they showed that HMGB1 was a late mediator of endotoxin-mediated sepsis in mice. Since this seminal finding, HMGB1 has been implicated in the pathogenesis of a variety of sterile inflammatory conditions including rheumatoid arthritis [95–97], lupus erythematosus, and Sjögren syndrome [98,99], trauma and hemorrhagic shock [100–103], and ischemia-reperfusion injury of the liver, heart, kidney, and brain [104–107], providing evidence for its role as a danger signal. It was recently shown that HMGB1 released from late apoptotic cells remains bound to nucleosomes and that HMGB1-nucleosome complexes activate antigen-presenting cells and induce secretion of cytokines by macrophages and expression of costimulatory molecules in DCs [108]. Because autoantibodies against double-stranded DNA and nucleosomes are a characteristic of systemic lupus erythematosus, HMGB1 bound to nucleosomes could therefore contribute to the pathogenesis of systemic lupus erythematosus.

In addition to studies highlighting the proinflammatory effects of HMGB1 in models of multiple diseases *in vivo*, there is emerging evidence to suggest that HMGB1 participates in tissue repair and remodeling, a role that is increasingly recognized as a characteristic of damage-associated molecules. HMGB1 is proangiogenic [109,110]. It induces migration of mesangioblasts [111,112], endothelial progenitor cells [112], and myogenic cells [113] and chemotaxis and proliferation of smooth muscle cells [114,115]. It stimulates myogenesis [116] and promotes myoblast differentiation [117] and myocardial regeneration after infarction [118]. It also promotes enhanced arteriole density, granulation tissue deposition, and accelerated wound healing in diabetic skin [119].

HMGB1 and Cancer. HMGB1 is widely expressed in many tumor cells and can be secreted by them or be released upon necrotic cell death [120,121]. Its expression is high in migrating growth cones and malignant cells [122]. It also binds tissue-type plasminogen activator and plasminogen, promoting plasmin production and hence tissue invasion [123,124]. Given its effects in tissue repair, wound healing, angiogenesis, and cell migration, HMGB1 could augment tumor growth and metastasis. In fact, studies suggest that up-regulation of HMGB1 is associated with a malignant phenotype of many cancers [125,126]. HMGB1 also mediates inflammation-based colon

carcinogenesis [127]. HMGB1 is significantly elevated in serum and colonic tissue during acute inflammation induced by DSS and anti-HMGB1 treatment reduces severity and extent of inflammatory lesions [127]. HMGB1 released by necrotic colon cells seems to affect surrounding inflammatory cells such as macrophages inducing inflammatory cytokine production and tissue repair. Apc/Min$^+$ mice, in which colon tumors were triggered by DSS-induced inflammation, also show a significant decrease in tumor numbers within the colon when treated with anti-HMGB1 [127]. Colon cancer–derived HMGB1 promotes growth inhibition and apoptosis of macrophages [128] suggesting a role for HMGB1 in the tumor microenvironment. Thus, targeting HMGB1 production or release, or blocking its interaction with its receptors and downstream signaling in macrophages, might have therapeutic applications in both inflammation and cancer [129]. However, as mentioned earlier, recent studies show that pulsed acute release of HMGB1 occurs after chemotherapy or radiotherapy [65], which promotes DC processing of apoptotic cells, DC maturation, clonal expansion of tumor-specific T cells, and antitumor immune response [66,130,131]. This suggests that HMGB1, depending on the pulsatile release from apoptotic tumor cells or chronic release from necrotic tumor cells, could have a paradoxical dual effect on tumors [67].

S100 Proteins

S100 proteins are a family of more than 20 homologous intracellular proteins characterized by calcium-binding EF hand motifs, low molecular weights, ability to form homodimers and heterodimers and oligomers, and tissue-specific expression [132–135]. Most of the S100 genes are clustered at the chromosomal region 1q21, a region frequently rearranged in epithelial tumors and tumors of soft tissues [132,135,136]. They have two distinct EF hand calcium-binding domains connected by a hinge region. The canonical C-terminal calcium binding EF hand is common to all EF hand proteins, whereas the N-terminal EF hand is characteristic of S100 proteins. Intracellular functions of S100 proteins have been extensively studied. These include calcium homeostasis, cell cycle regulation, cell growth and migration, cytoskeletal interactions, protein phosphorylation, and regulation of transcriptional factors among others [132–135].

Extracellular Functions of S100 Proteins. Extracellular functions have been reported for a few S100 proteins. The most well studied extracellular effects relate to the myeloid-specific S100 proteins, namely, S100A8, S100A9, and S100A12 [133,137,138]. They are expressed predominantly in cells of myeloid origin. S100A8 and S100A9 are present in neutrophils, monocytes, and myeloid progenitors and can be induced in keratinocytes during inflammation [136]. Expression is downregulated during macrophage and DC differentiation [139–141]. S100A12 expression is restricted to neutrophils and is not expressed in rodents [142,143]. Like other DAMP molecules, the proteins lack secretion signals required for classic Golgi-dependent transport and are released by an energy-dependent and tubulin-dependent process, which requires activation of protein kinase C [144]. When secreted into the extracellular medium in response to cell damage or activation, they become danger signals that activate other immune cells and endothelial cells [138].

Multimeric forms of S100 proteins seem to be necessary for the extracellular functions of S100 proteins [145,146]. Multimeric assemblies have been reported for S100A12, S100A4, and S100B. S100A8 and S100A9 function predominantly as S100A8/A9 heterodimers. Disruption of the *S100A8* gene causes late embryonic lethality [147], whereas S100A9 null mice do not exhibit an obvious phenotype. However, targeted deletion of S100A9 leads to a complete lack of S100A8 and a functional S100A8/A9 complex in peripheral blood cells and cells of the bone marrow, despite normal mRNA levels of S100A8, suggesting that S100A9 expression is important for the stability of the S100A8 protein [148,149].

S100 Proteins and Inflammation. S100A8/A9 and S100A12 induce prothrombotic and proinflammatory responses in endothelial cells including induction of thrombospondin, chemokines, and adhesion molecules and stimulate proinflammatory cytokine production by macrophages [142,150–154]. Up-regulation of chemokines and adhesion molecules helps to promote further recruitment of leukocytes into inflamed tissues. S100A8/A9 and S100A12 are elevated early in tissues and serum in many pathological conditions associated with inflammation such as arthritis, inflammatory bowel disease, vasculitis, multiple sclerosis, psoriasis, and cystic fibrosis and are considered suitable biomarkers of inflammation [133,137,138].

S100A8/A9 and Cancer. It is becoming increasingly clear that S100A8/A9 proteins are involved in many aspects of tumor growth and metastasis. They are upregulated in many cancers including lung, gastric, colorectal, prostate, breast, and pancreatic cancers [136,155]. At low concentrations, S100A8/A9 promote tumor cell growth [19,156]. Elevated levels of S100A8/A9 in chronic inflammation and cancer suggest that the proteins play important roles in inflammation-mediated carcinogenesis.

Recent studies show that S100A8/A9 regulate the accumulation of MDSC in tumors [141,157]. S100A8 and S100A9 are downregulated during normal differentiation of myeloid precursors to DC and macrophages [139–141]. However, tumor-derived factors promote sustained up-regulation of S100A9 in myeloid precursors, which results in the inhibition of differentiation to DC and accumulation of MDSC [141]. These tumor-induced effects are not observed in cells from S100A9 null mice, which show less accumulation of MDSC, higher rate of tumor rejection, and lower tumor size than wild-type controls. This study also shows that activated STAT3 upregulates the expression of S100A8 and S100A9 in myeloid cells *in vitro* and *in vivo*. In a parallel study, we reported not only that S100A8/A9 are synthesized and secreted by MDSC but also that they have binding sites for S100A8/A9 [157]. Part of the binding is mediated by carboxylated glycans and by RAGE, leading to intracellular NF-κB signaling and MDSC migration. These findings strongly suggest that the S100A8/A9 proteins support an autocrine feedback loop that sustains accumulation of MDSC in tumors, alongside IL-6, IL-1β, prostaglandin E_2, and complement components [158].

S100A8/A9 are also involved in early metastatic processes. Soluble factors such as vascular endothelial growth factor, transforming growth factor β, and TNFα expressed by primary tumors and/or TAMs induce expression of S100A8 and S100A9 in myeloid and endothelial cells of premetastatic lungs [159]. These changes in the local microenvironment termed "premetastatic niche" represent early events in tumor dissemination and dictate the pattern of metastasic spread [160]. Expression of S100A8/A9 in myeloid and endothelial cells in the lung promote homing of tumor cells to these premetastatic niches [159]. If the tumor cells encounter myeloid progenitors at the premetastatic sites, this could promote an angiogenic switch necessary for metastatic cell survival. These studies indicate that S100A8/A9 could be targeted to prevent tumor metastasis. Other S100 proteins have also been implicated in

cancers, including S100B, S100A4, S100A7, S100A11, and S100P, and have been the subject of recent reviews [155,161].

Receptors That Detect Endogenous Danger Signals

It is becoming increasingly evident that intracellular mediators released upon necrotic cell death elicit inflammatory responses through recognition by signaling receptors, similar to the recognition of PAMPs. In fact, emerging evidence shows that some of the same PRRs that recognize PAMPs may also mediate responses to endogenous danger signals. By recognizing either pathogens or danger signals, PRRs seem to represent a common pathway to alert the host of danger and to promote tissue repair and regeneration.

Toll-like Receptors

Toll-like receptors or TLRs are a family of transmembrane receptors that recognize microbial molecular patterns or PAMPs and enable cells of the innate immune system to mount inflammatory responses against pathogens [59,162]. Different microbial moieties signal through different TLRs. Lipopolysaccharide from gram-negative bacteria is recognized by TLR4; double-stranded RNA activates TLR3; bacterial flagellin stimulates TLR5. TLR1, TLR2, and TLR6 recognize bacterial peptidoglycans, lipoproteins, lipoteichoic acids, lipoarabinomannan, and yeast zymosan; TLR7 recognizes single-stranded RNA; unmethylated CpG motifs in DNA are recognized by TLR9. Toll-like receptors that recognize viral nucleic acids such as TLR3, TLR7, and TLR9 are localized in endolysosomal compartments, whereas those that recognize bacterial protein and lipid ligands are expressed on the cell surface. All TLRs except TLR3 associate with myeloid differentiation factor 88 (MyD88), and this stimulates a kinase cascade resulting in the activation of mitogen-activated protein kinases (MAPKs), c-Jun N-terminal kinases, p38, and extracellular signal–regulated kinases, and NF-κB [163,164].

In addition to PAMPs, TLR2 and TLR4 also recognize endogenous danger signals [165,166]. HMGB1 binds to TLR2 and TLR4 [167,168]. The ability of HMGB1 to stimulate NF-κB activation and cytokine production in macrophages and to promote neutrophil recruitment *in vivo* in response to inflammation is dependent in part on TLR signaling pathways [103,169–171]. HMGB1 released by chemotherapy-induced cell death binds to TLR4 and induces anti-tumor T-cell immunity [65]. HMGB1 bound to nucleosomes from apoptotic cells induces anti-dsDNA and anti-histone immunoglobulin G responses in a TLR2-dependent manner [108]. HMGB1-RAGE interaction acts in a costimulatory manner for TLR9-mediated responses to DNA-containing immune complexes [91,92].

S100A8/A9 were recently shown to interact with TLR4, promoting endotoxin-induced shock [172]. The ability of S100A8 and S100A9 to promote premetastatic niches in lungs also requires TLR4-mediated signaling [173]. They induce serum amyloid A (SAA3) expression in premetastatic lungs, which attracts myeloid cells to the premetastatic niches. SAA3 stimulates TLR4 activity and promotes NF-κB activation [173]. S100A8/A9-SAA3-TLR4 paracrine cascade mediated through NF-κB could therefore be involved in early pulmonary metastasis.

Ligation of TLRs lead to several host defense events that protect the host from infection and damage induced injury [162,174]. TLRs promote tissue repair and regeneration through their angiogenic and antiapoptotic effects [64,174]. MyD88 is essential for the promotion of diethylnitrosamine-induced hepatocellular tumors, spontaneous and azoxymethane-induced intestinal tumorigenesis, and chemically induced skin tumors [175–177]. TLR4 signaling also promotes colitis-induced colon carcinogenesis [178]. Ligands involved in TLR-mediated tissue regeneration and carcinogenesis are unknown. It is likely that TLRs are activated by microbial entities in the gut and enterohepatic circulation or by endogenous ligands such as HMGB1, S100A8/A9, or extracellular matrix components released by necrotic cell death.

Receptor for Advanced Glycation End Products

RAGE, originally discovered as a receptor for advanced glycation end products (AGE), is a multiligand receptor of the immunoglobulin superfamily that plays a key role in immune and other signaling responses mediated by HMGB1 and many S100 proteins [161,179, 180]. RAGE also binds other structurally unrelated ligands such as amyloid β peptide, transthyretin, and Mac-1 integrin. It is expressed on monocytes, macrophages, T cells, DCs, smooth muscle cells, immature myofibers, endothelial cells, embryonal neuronal, and tumor cells. Expression is high during embryonic development and low in healthy adult tissues, except in the lung where it is constitutively expressed at high levels [181]. RAGE is implicated in multiple pathologies including diabetes, inflammation, neuronal degeneration, and cancers, primarily as a receptor for DAMPs [161,182–184].

RAGE contains a single variable (V) domain containing two N-glycosylation sites, followed by two constant (C1 and C2) domains, a transmembrane segment and a short cytoplasmic tail necessary for ligand-induced signal transduction [185]. Most ligands bind to the V domain. Different RAGE splice variants exist and have recently been classified as RAGE, RAGE_v1 to RAGE_v19 [186]. The prevalent isoforms are full-length RAGE (RAGE), secreted RAGE that lacks the cytoplasmic and transmembrane domain (sRAGE, RAGE-v1), and N-terminal truncated RAGE (RAGE-v2). The relative expression of the isoforms is tissue-specific. RAGE-v1 or sRAGE is believed to regulate full-length RAGE activation through its ability to bind ligands extracellularly.

HMGB1 and RAGE

RAGE was the first identified receptor for HMGB1 [187]. RAGE-HMGB1 interactions mediate NF-κB–dependent production of cytokines and up-regulation of cell surface receptors [171]. HMGB1 stimulates endothelial progenitor cell migration to ischemic and tumor regions in a RAGE- and integrin-dependent manner [112] and RAGE mediates the proangiogenic effects of HMGB1 [109]. Effects of HMGB1 on mesangioblast homing, skeletal muscle regeneration, chemotaxis of smooth muscle cells, and myogenesis are partly mediated by RAGE [111,113,114,116]. RAGE-HMGB1 interactions also promote DC maturation, homing, and T-cell activation [79,80].

HMGB1-induced RAGE signaling also mediates embryonal neurite outgrowth [187]. This finding, combined with expression of HMGB1 at the leading edges of motile cells and ability to bind tissue-type plasminogen activator and plasminogen leading to the production of plasmin [122,123,188], suggested that HMGB1-RAGE interactions could promote tumor invasion and metastasis. In fact, overexpression of HMGB1, along with RAGE, has been associated with proliferation and metastasis of many tumors [120,124,128,189,190]. However, the tumorigenic effects may be tissue- and cell-dependent because the expressions of RAGE and HMGB1 and their interaction have also been shown to correlate negatively with tumor growth. For example, RAGE is constitutively expressed in the lung, and down-regulation of RAGE and HMGB1 is associated with increased aggressiveness of lung carcinomas [191]. RAGE-HMGB1 engagement also reduces tumor potential of rhabdomyosarcoma cells *in vitro* and *in vivo*, suggesting that reduced RAGE signaling may contribute to rhabdomyosarcomagenesis

[117,192]. In patients with esophageal and oral squamous cell carcinomas, reduced expression of RAGE negatively correlates with tumor invasion and associated with better prognosis [193,194]. Soluble RAGE and HMGB1 are expressed in tumors of cartilage. Whereas RAGE expression correlates positively with tumor grade and survival, HMGB1 expression does not, suggesting distinct functions of the soluble form of RAGE and HMGB1 [195].

S100 Proteins and RAGE

S100B and S100A12 were the first of the S100 proteins shown to initiate intracellular signaling through interaction with RAGE [152,196]. Since then, a large number of S100 proteins have been shown to bind to RAGE, and some of these promote inflammation and cancer [145,146,155,161]. S100 proteins and RAGE are coexpressed in a variety of human tumors [197]. Like many S100 and DAMP proteins, S100B exhibits both intracellular and extracellular functions [198]. Intracellular S100B stimulates cell proliferation and migration and inhibits apoptosis and differentiation, whereas extracellular S100B exerts regulatory effects on a relatively larger number of cell types in an autocrine and paracrine manner through RAGE and possibly other receptors [198]. S100B is also implicated in diabetes and inflammation. S100B induces RAGE-dependent inflammatory gene expression and oxidative burst in monocytes, macrophages, microglia, and neurophils at high concentrations that could be relevant in local inflammatory environments in both acute and chronic inflammations [198]. S100B causes chemoattraction of RAGE-expressing encephalitogenic $CD4^+$ T_H1 T cells in a model of experimental autoimmune encephalomyelitis suggesting a role in the pathophysiology of multiple sclerosis [199]. S100B promotes RAGE-dependent activation of NF-κB in endothelial cells, inducing expression of vascular cell adhesion molecule 1, macrophage chemotactic protein 1, and RAGE [200,201]. It also triggers signaling pathways in smooth muscle cells in a RAGE-dependent manner, resulting in the up-regulation of macrophage chemotactic protein 1 and IL-6 [202]. Interaction of S100A7 or Psoriasin with RAGE mediates chemotaxis of leukocytes [203]. S100A8/A9 are upregulated in many inflammatory diseases, and RAGE and S100A8/A9 are coexpressed in tumors [156,204,205] and are linked to downstream signaling in tumor cells and endothelial cells [153,156,204]. Recent studies provide a more direct evidence of the interaction of S100A8/A9 to RAGE [19,156,206]. At low concentrations, S100A8/A9–induced NF-κB activation promote the growth of tumor cells. This effect is blocked by RAGE gene silencing or by treatment with anti-RAGE [156]. S100A8/A9 also promote LPS-induced cardiac myocyte dysfunction and RAGE coimmunoprecipitates with S100A8 and S100A9 suggesting a direct role for RAGE in S100A8/A9–mediated effects in cardiac myocytes [206]. More recently, we showed that S100A8/A9 binds to a subpopulation of RAGE modified by carboxylated glycans [19] suggesting a direct interaction between the proteins. S100A11 has been shown to modulate osteoarthritis through interaction with RAGE [207]. S100A12-mediated RAGE activation has been implicated in colon inflammation [152]. Functional interactions of RAGE and S100P in pancreatic and colon cancer cells have been demonstrated [208,209].

As mentioned above, most RAGE ligands, including AGEs, HMGB1, S100 proteins, and amyloid β peptide, are highly elevated in inflammatory foci, and RAGE-dependent inflammation promotes up-regulation of both ligands and receptor leading to a feed-forward signaling [182,210], amplifying the inflammatory environment that would promote tumorigenesis. In support of this, recent studies indicate a role for RAGE in inflammation-induced carcinogenesis. RAGE null mice are resistant to the onset of DMBA/TPA–induced skin carcinogenesis and azoxymethane/DSS–induced colon carcinogenesis [18,19]. In both these models, S100A8/A9 are strongly upregulated in stromal cells within the tumors. $RAGE^{-/-}$ mice show reduced levels of MDSC in the DMBA/TPA–induced skin carcinogenesis, implicating RAGE in S100A8/A9–induced MDSC recruitment [18].

Other Cell Surface Binding Sites for DAMP Molecules

DAMP molecules HMGB1, S100A8/A9, and S100A12 bind to a novel modification of N-glycans called carboxylated glycans, which are expressed on RAGE and other glycoproteins [211,212]. HMGB1 binds heparan sulfate proteoglycans, heparin, syndecan, and phosphocan [122,213]. S100A8/A9 also bind to heparan sulfate proteoglycans [214].

Signaling Pathways Activated by DAMP Ligation Converge on NF-κB

RAGE ligation by DAMPs leads to the activation of signaling pathways (Erk1/2 MAPKs, Cdc42/Rac SAP/JNK, and p38 MAPKs) implicated in cell proliferation and cell migration [124,152,215,216]. Toll-like receptors can signal through MyD88, IL-1 receptor–associated kinase, TNF receptor–associated factor, Akt, Cdc42/Rac, phosphatidyl inositol-3 kinase, and MAPKs [163,167,172,217]. Signaling pathways activated by DAMP ligation of the PRRs result in activation of NF-κB [218,219], which further promotes the expression of proinflammatory cytokines, chemokines, angiogenic factors, adhesion molecules, nitric oxide synthase, matrix metalloproteases, and antiapoptotic genes [29]. Chronic NF-κB activation and subsequent inflammation, angiogenesis, tissue repair, and regeneration could therefore lead to tumor development. In fact, specific inactivation of the classic NF-κB activation pathway in epithelial cells and macrophages reduces the formation of inflammation-associated colonic tumors in mice, suggesting that sustained NF-κB activation in either or both of these cells may provide a critical link between inflammation and cancer [17,29]. TNFα-induced NF-κB activation also promotes hepatitis-associated carcinoma in Mdr2 null mice [30].

Relative Importance of the Different Receptors and DAMP Molecules in Inflammation and Cancer, and Binding Specificities

PRRs represent a limited number of proteins by which cells recognize microbial entities and endogenous danger signals and orchestrate an immune response. However, the relative importance and contribution of the different DAMP molecules and of RAGE, TLRs, and other receptors in mediating inflammation and cancer are not completely understood and are likely to differ between cell types. If we learn more about the specificity of these interactions, we can also determine targets for inhibition. Binding specificity may be imparted by interactions of different domains on the ligands and receptors. The epitopes on DAMPs recognized by RAGE might be different from those recognized by TLRs. HMGB1 may interact with distinct receptors through its DNA-binding boxes or through the C-terminal domain. For example, amino acids 150 to 183 of HMGB1 interact with RAGE [220]. Similarly, distinct epitopes on RAGE and TLRs recognized by the ligands may also impart specificity. For example, it is becoming increasingly clear that different S100 proteins require different domains on RAGE for binding. S100A12 binds to V-C1 domains, S100B requires the V domain of RAGE and S100A6 interacts with V-C2 domain [221–224]. Posttranslational modifications such as glycosylation on the

receptors, acetylation, or phosphorylation of ligands and formation of multimolecular assemblies as with S100 proteins could also play important roles in defining specificity of interactions and downstream signaling.

Our studies suggest that N-glycan modifications of RAGE may serve as unique ligand binding sites and may contribute to some of the pleiotropic binding ability of the receptor. RAGE has two N-glycosylation sites on the ligand binding V domain and both sites are occupied by complex and hybrid N-glycans. Our recent analysis of N-glycans on sRAGE suggests considerable heterogeneity of glycan structures on RAGE (Houliston et al., unpublished data). Several years ago, we identified a novel group of anionic N-glycans that contain an immunogenic carboxylate group unrelated to sialic or uronic acids [225]. These carboxylated glycans contain glutamic or aspartic acids [226]. Using a monoclonal antibody against the glycans (mAbGB3.1), we found that the glycans show restricted expression on mouse and human cells of myeloid lineage including monocytes, macrophages, and DCs and on endothelial cells [227,228]. They are absent or undetectable on normal epithelial cells. However, they are expressed on several tumor cells [19]. To identify glycan-binding proteins, we applied whole bovine lung homogenates through a column of carboxylated glycans and found that DAMP molecules HMGB1, S100A8/A9, and annexin I specifically bound to the column [211,212]. We found that a subpopulation of RAGE molecules is modified by carboxylated glycans [19,212] and that binding of HMGB1 to RAGE partially depends on carboxylated glycans [212]. The subpopulation of RAGE enriched for carboxylated glycans by mAbGB3.1 also showed 10- to 100-fold increase in binding potential (B_{max}/K_d) for both S100A8/A9 and S100A12, suggesting that carboxylated glycans form critical binding sites for these ligands on RAGE. Conversely, based on our recent unpublished findings, S100A11, S100B, and S100A1 as well as AGE do not show enhanced binding to mAbGB3.1-enriched RAGE, suggesting that, although many S100 family members bind RAGE, they may bind to different structural

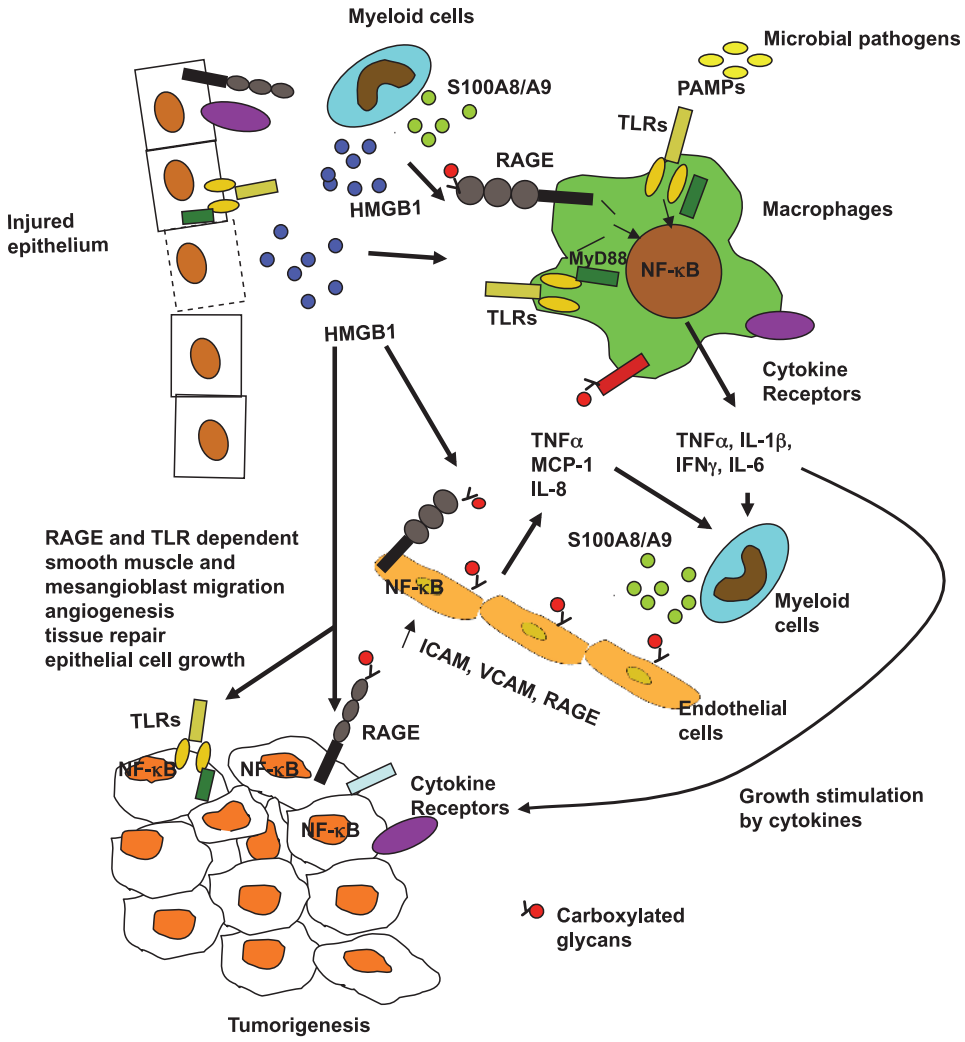

Figure 1. Damage-associated molecular pattern molecules in inflammation. Inflammation can be initiated by microbial PAMPs or by intracellular danger signals such as HMGB1 and S100A8/A9 released from necrotic cells or secreted from monocytes. HMGB1 and S100A8/A9 bind to TLRs or RAGE and promote NF-κB signaling and expression of cytokines that act as growth factors for tissue repair and regeneration functioning through their respective receptors (generic representation for cytokine receptor(s) shown here). HMGB1 and S100A8/A9 promote expression of adhesion molecules and cytokine production by local vascular endothelium, which further attract neutrophils and monocytes. HMGB1 also promotes RAGE- and TLR-dependent smooth muscle and mesangioblast migration, angiogenesis, and tissue repair. Nuclear factor-κB–dependent proinflammatory cytokines in turn upregulate the expression of DAMPs and RAGE, leading to a pathological cycle of inflammation, necrosis, and tumorigenesis.

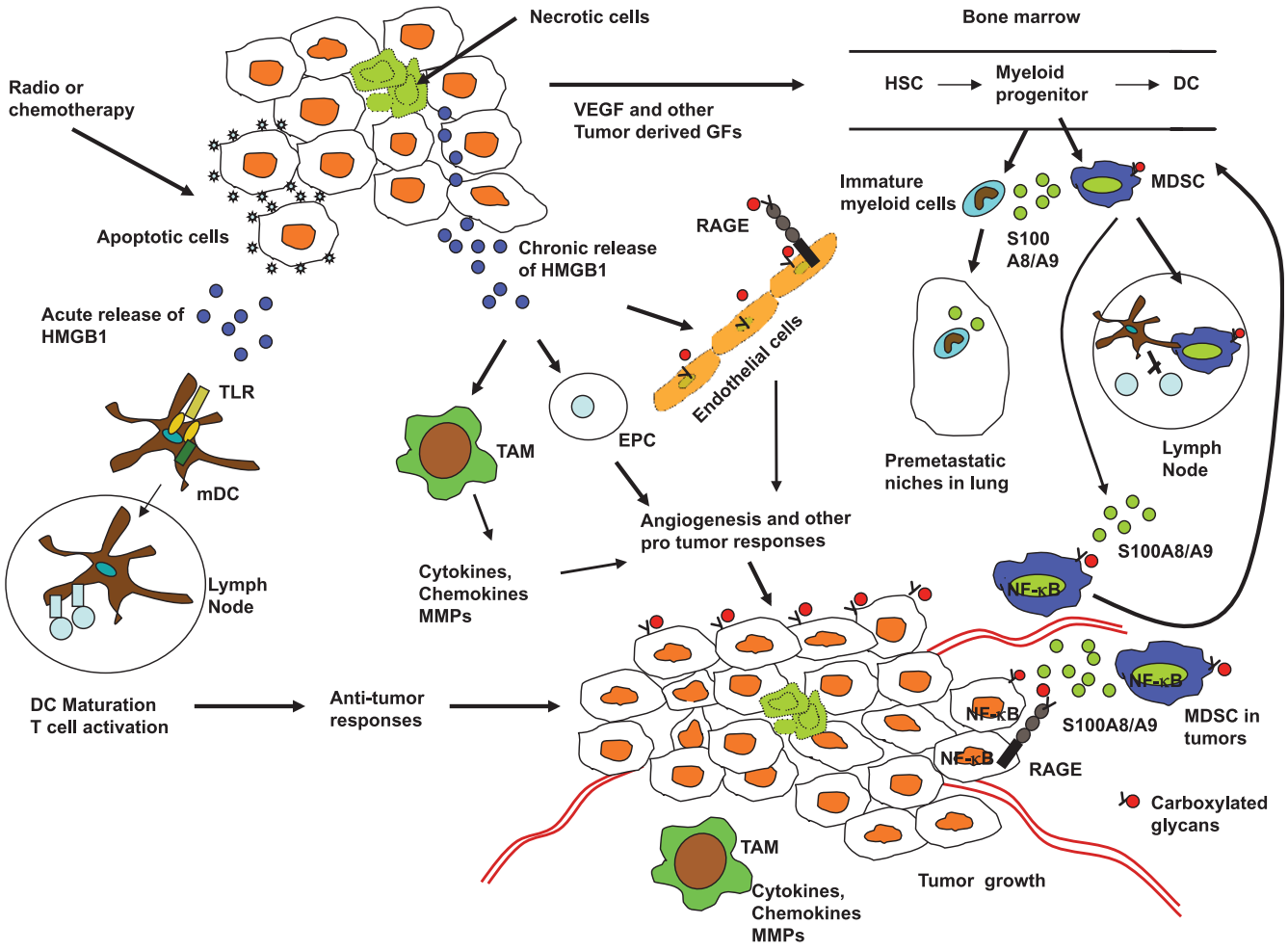

Figure 2. Damage-associated molecular pattern molecules in the tumor microenvironment. HMGB1 has a dual effect on tumors. Acute release of HMGB1 after antitumor treatments promotes maturation of DCs through interaction with TLR4 and clonal expansion of tumor antigen–specific T cells and antitumor responses. Conversely, persistent hypoxia in growing tumors leads to necrosis, causing chronic release of HMGB1, which activates protumor responses promoting angiogenesis and tumor growth through the recruitment of macrophages (TAM) and endothelial precursor cells (EPC) and activation of local endothelial cells through RAGE signaling. In the bone marrow, S100A8/A9 are downregulated during normal differentiation of myeloid precursors to DC and macrophages. However, tumor-derived factors promote sustained up-regulation of S100A9 in myeloid precursors through a STAT3 dependent process, which results in the inhibition of DC differentiation and accumulation of MDSC. S100A8/A9 are synthesized and secreted by MDSC and bind carboxylated glycans on other MDSC. This promotes migration and accumulation of MDSC in blood and peripheral lymphoid organs, possibly through RAGE- and NF-κB–dependent pathways, thereby establishing an autocrine feedback loop that maintains MDSC levels and promoting immune suppression against tumors. S100A8/A9 promote tumor growth through RAGE- and carboxylated glycan–dependent pathways. Tumor-derived factors also induce expression of S100A8/A9 in myeloid and endothelial cells in premetastatic niches within lungs, which promotes homing of tumor cells to lungs.

domains or regions on the receptor, some involving carboxylated N-glycans on the V domain and others not, thus providing differential binding specificity. In support of this, we found that the mAbGB3.1-enriched population of RAGE forms higher-order complexes of S100A12 and that deglycosylation of RAGE reduced the ability to form multimeric complexes.

As further evidence of the importance of carboxylated glycans in mediating DAMP interactions, we found that inhibiting carboxylated glycan-dependent interactions of DAMP molecules using mAbGB3.1, blocked onset of T-cell–mediated colitis [228], colitis-dependent colon cancer [19], and recruitment of MDSC to secondary lymphoid organs and accumulation of MDSC in blood [157]. Proteomic analysis (MudPIT) of mAbGB3.1-immunoprecipitated proteins from macrophages revealed the presence of RAGE among other glycoproteins, but not TLR2 or TLR4, suggesting that this modification may not be present on all PRRs (Srikrishna et al., unpublished data). Our studies on colitis-associated carcinogenesis also show that S100A8/A9 and HMGB1 could participate in distinct events in disease progression through different receptors [19]: an acute inflammation phase involving TLR4 and a tumorigenesis and progression phase involving the glycans and RAGE expressed on tumor cells because mAbGB3.1 does not block early DSS-mediated colitis but blocks chronic inflammation and carcinogenesis. In addition, $RAGE^{-/-}$ mice are as susceptible to early DSS-induced injury as $RAGE^{+/+}$ mice but are resistant to colitis-mediated cancer.

Current structural studies on RAGE are performed on the extracellular domains (VC1C2) that comprise soluble sRAGE but are often

produced by expression systems (bacteria, insect cells, or yeast) that lack the complex glycosylation machinery of the mammalian systems. Structural and ligand-binding analysis is typically done on single or on tandem domains [221]. Nuclear magnetic resonance analysis of expressed VC1 domains show that they form an integrated structural unit that binds to Ca^{2+}-S100B [223]. One of the critical residues in the interactive face (Thr27) is part of the unoccupied N-glycosylation sequon; the presence of a normal glycan chain on a native RAGE protein on cells would likely alter this interaction. S100A12 is shown to bind to the C1 domain of RAGE [221]; however, the protein used in this study has no N-glycans because it was expressed in *Escherichia coli*. On the basis of our findings, it is likely that both V and C1 domains are necessary for binding to S100A12. AGEs bind to the V domain [229], whereas S100A6 binds to the C1C2 domains [222]. K_d estimates for S100 ligands range from 5 to 500 nM depending on the analytical methods, specific ligands, and conditions. All studies concur that RAGE-ligand binding generates multimeric complexes of both RAGE (tetramer) and ligands (tetramer, hexamer, octomer) and that formation of these higher-order complexes may be essential for signal transduction [145, 224,230]. The efficiency of complex formation varies widely, sometimes requiring extremely high concentrations of protein (500 mM) and involving only a few percent of the molecules. It is unclear whether the *in vitro* conditions mimic the formation of signaling-competent RAGE-ligand multimers on a cell surface. Glycans are often regarded as impediments in high-resolution protein structural analysis, but their role in formation of signaling complexes is now well documented [231,232]. Studies on glycan-deficient RAGE, therefore, may not accurately reflect the appropriate *in vitro* or *in vivo* ligand binding, domain interactions, complex formation, or details of the signaling pathways. Detailed binding studies using fully glycosylated RAGE protein are therefore necessary.

Figures 1 and 2 provide a representation of the current findings on the role of DAMP molecules and PRRs in mediating inflammation and cancer.

Conclusions and Future Perspectives

Recent studies show that chronic inflammation and necrotic cell death contribute to tumorigenesis. These studies provide novel insights into the functional role of danger signals such as HMGB1 and S100 proteins released during necrotic cell death and inflammation and the receptors that detect them such as TLRs and RAGE in mediating the pathology. Signaling responses mediated by DAMP molecules include production of cytokines and chemokines, recruitment of leukocytes such as MDSC and associated immune suppression, neoangiogenesis, stromagenesis, and epithelial proliferation. These represent homeostatic tissue repair and remodeling responses and have implications for carcinogenesis when chronic inflammatory states and necrotic cell death lead to uncontrolled responses. Studies suggest therapeutic strategies based on blocking the interactions of DAMP molecules and their receptors or downstream signaling pathways. These include administration of sRAGE, antibodies to RAGE, TLRs, HMGB1, S100 proteins, or carboxylated glycans, and inhibitors of MAPK pathways, NF-κB, and other signaling mediators. Recent studies suggest that pulsed release of HMGB1 after chemotherapy and radiotherapy in fact triggers antitumor immune responses. Further studies are therefore clearly needed to define which set of intracellular molecules constitute danger signals, understand specificity of interactions, whether early and late events are driven by different PRRs, what controlled stimuli could promote apoptosis and antitumor responses, and if inflammatory response to tissue injury can be selectively inhibited without affecting normal host defense mechanisms.

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